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Efficient manipulation of the human adenovirus genome as an infectious yeast artificial chromosome clone

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ABSTRACT **A yeast artificial chromosome (YAC) containing a complete human adenovirus type 2 genome was constructed, and viral DNA derived from the YAC was shown to be infectious upon introduction into mammalian cells. The adenovirus YAC could be manipulated efficiently using homologous recombination-based methods in the yeast host, and mutant viruses, including a variant that expresses the human analog of the *Saccharomyces cerevisiae CDC27* gene, were readily recovered from modified derivatives of the YAC. The application of powerful yeast genetic techniques to an infectious adenovirus clone promises to significantly enhance the genetic analysis of adenovirus and to simplify the construction of adenovirus-based vectors for vaccines or for gene transfer to mammalian cells or whole animals. The adenovirus YAC was produced by homologous recombination *in vivo* between adenovirus 2 virion DNA and YAC vector plasmids carrying segments of the viral left and right genomic termini. This recombinational cloning strategy is generally applicable to the construction of YACs containing other DNA segments, such as the genomes of other viruses. Further, it is very efficient and may permit the targeted cloning of segments of the genomes of higher organisms directly from genomic DNA.**

Adenoviruses are among the most widely exploited experimental model systems for studies of basic eukaryotic molecular biology. The splicing of eukaryotic mRNAs was first observed in studies of adenoviruses (1), the *in vitro* adenoviral DNA replication system was the first for a eukaryotic replicon (2), and binding of the product of the *RB* gene to a viral oncoprotein was first demonstrated in adenovirus-infected cells (3). Adenoviruses also promise to be valuable as vectors for gene transfer to whole organisms or to individual cells for the purposes of vaccination and gene therapy. For example, an adenovirus-based recombinant rabies vaccine has been shown to be efficacious in dogs (4), and adenovirus-based gene transfer vectors have been used for the transfer of a human gene to the lung epithelium of rats (5).

The study of basic adenoviral biology and the development of adenoviral vectors both require manipulation of the viral genome: in the first case for the production of viral mutants for genetic studies and in the second to incorporate exogenous DNA into the viral genome and to optimize its expression. Manipulation of adenoviral DNA is possible using current *in vitro* and *Escherichia coli*-based technology, but the schemes employed are time-consuming. In contrast, a variety of methods are available for the rapid manipulation of large DNA segments in yeast. To make it possible to apply yeast genetic methods to the adenoviral genome, we have constructed in *Saccharomyces cerevisiae* a yeast artificial chromosome (YAC) that contains a complete copy of the linear 36-kb adenovirus type 2 (Ad2) genome. The cloned adenoviral genome is infectious, the viral sequences can be modified efficiently using conventional yeast genetic techniques and vectors described below, and mutant viruses can be recovered from modified YAC clones. Remarkably, the recombinational strategy used to construct the adenovirus YAC proved to be very efficient. Our data suggest that it will be of use in the targeted cloning of specific genomic DNA segments from higher organisms.

MATERIALS AND METHODS

Plasmids. The YAC vector pair pRML1 and pRML2 are described in Spencer *et al.* (6). Together, these plasmids contain the cis-acting genetic elements required for YAC maintenance in yeast (telomeres, a centromere, and origins of replication), genetic markers used to select yeast cells containing the YAC (*TRP1* and *URA3*), and a system derived from pCGS966 (7) that permits amplification of the YAC by growth in selective medium (a conditional centromere and the herpes simplex virus thymidine kinase gene under the control of the yeast *DED1* promoter). pRML1Ad2L was made by inserting a segment of the left end of the Ad2 genome between *Eco*RI and *Cla* I sites of pRML1; pRML2Ad2R was made by inserting a segment of the right end of the Ad2 genome between the *Eco*RI and *Bgl* II sites of pRML2. Both viral fragments were produced from Ad2 virion DNA by PCR. The viral portion of the genomic terminal primer used to amplify both fragments (5′-CCGAATTCTACGTACATCATCAATAATATACC-3′; adenoviral sequence is underlined) differed from the published Ad2 sequence (8) by the insertion of an adenosine residue (boldface type) between positions 7 and 8, and the primer contained both a *Sna*BI site immediately adjacent to the viral terminal sequence and an *Eco*RI site used for cloning. The internal primer used to produce the left-hand fragment included Ad2 nt 988–1005; the primer used to produce the right-hand fragment covered Ad2 nt 34,357–34,374. The *Cla* I and *Bgl* II sites used for cloning occur naturally in Ad2 DNA at nt 916 and 34,390, respectively.

p680, an integrating plasmid containing the *LEU2* gene and the dominant cycloheximide-sensitivity allele (*CYH2*^{r}) of *CYH2* (9), is described in Spencer *et al.* (6). p680E3Δ was constructed by insertion of two PCR fragments (Ad2 nt 27,410–28,404 and 30,801–31,825) between *Xho* I and *Sal* I sites and between *Xba* I and *Sac* II sites, respectively, in the polylinker of p680. For construction of H2*sub*CDC72Hs, a cDNA copy of the CDC27Hs gene (10) fused at its 3′ end to sequences encoding two repetitions of the C-terminal 28 amino acids of the avian infectious bronchitis virus E1 glycoprotein (11) was inserted between the *Sal* I and *Xba* I sites in p680E3Δ. Construction details of the epitope-tagged version of CDC27Hs will be presented elsewhere.

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Abbreviations: YAC, yeast artificial chromosome; Ad2, adenovirus type 2; E3, early region 3.

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Yeast Methods. Yeast selective media are described in Rose *et al.* (12). For the preparation of YACs, spheroplasts of the yeast strain YPH857 (*MATα, ade2-101, cyh2r, leu2Δ1, lys2-801, his3Δ200, trp1Δ63, ura3-52*; ref. 6) were transformed with equimolar quantities of Ad2 virion DNA, pRML1Ad2L linearized with *Cla* I, and pRML2Ad2R linearized with *Bgl* II (10 μg, total). For two-step gene replacements, YAC-containing strains were transformed by the lithium acetate method. Detailed protocols are presented in Spencer *et al.* (6). Amplification of the adenovirus YAC was by growth of YAC-containing strains in selective medium containing thymidine (800 μg/ml), sulfanilamide (1 mg/ml), and methotrexate (10 μg/ml), with galactose as the carbon source (7). Cultures were inoculated with 10^5 cells per ml; growth to saturation took 3–5 days at 30°C. Amplified cultures were diluted 1:50 into YPD and grown overnight before analysis by pulsed-field gel electrophoresis or preparation of DNA for transfection. High molecular weight yeast DNA was prepared by a protocol developed by A. Wilmen, M. Funk, and J. Hegemann (personal communication). Cells were collected by centrifugation from 500-ml YPD cultures of cells at 5×10^7 cells per ml, washed once in water, suspended in 20 ml of 1 M sorbitol/4.25 mM KH_2PO_4/42.75 mM K_2HPO_4/20 mM dithiothreitol, and incubated for 1 h at 37°C. Cells were collected and incubated for 1 h at 37°C in 20 ml of 1 M sorbitol/1 mM EDTA (DB) containing zymolyase 20T (200 μg/ml). The resulting spheroplasts were collected, washed once in DB, resuspended in 15 ml of 20 mM Mops/0.84 mM spermine/2.16 mM spermidine/10 mM K_2EDTA/2 mM EGTA/1 mM aminoacetonitrile/1 mM phenylmethylsulfonyl fluoride/0.2% Triton X-100/1% thiodiglycol, and disrupted by five strokes of a glass Potter B type homogenizer. Undisrupted spheroplasts were removed by centrifugation at 120 × *g* for 10 min; nuclei were collected by centrifugation at 3000 × *g* for 10 min, resuspended in 0.5 ml of 20 mM EDTA/1% SDS/20 mM Tris·HCl, pH 8.0/proteinase K (50 μg/ml), and incubated at 65°C for 2–5 h. Potassium acetate (0.5 ml of 5 M) was added and the samples were placed at 4°C for 30 min. The precipitate was removed by centrifugation, and nucleic acids were precipitated from the supernatant by addition of an equal volume of isopropanol. The pellet was gently dissolved in 10 mM Tris·HCl, pH 8.1/1 mM EDTA (TE) containing RNase A (200 μg/ml) and incubated for 2 h at 37°C; DNA was precipitated with ethanol and gently dissolved in TE.

RESULTS

Recombinational Cloning of the Adenovirus Genome. Adenovirus YACs were constructed by recombination *in vivo* between Ad2 virion DNA and linear forms of the YAC vector plasmids pRML1Ad2L and pRML2Ad2R after transformation of the three DNAs into yeast spheroplasts (Fig. 1*A*). After transformation, *TRP1 URA3* transformants were selected, colony-purified, and examined to identify YAC-containing strains.

In strains that contain an adenovirus YAC, the *TRP1* and *URA3* genes will be linked, and as a consequence, *URA3* will be retained under conditions that select for *TRP1*. To test the stability of *URA3*, transformants were plated on medium that selects for *TRP1* and the resulting colonies were replica-plated on medium that selects for *TRP1* and against *URA3* [with 5-fluoroorotic acid (FOA) at 1 mg/ml (13)]. These conditions reveal transformants in which *URA3* can be lost while *TRP1* remains: such strains will produce FOA-resistant (*TRP1, ura3*) segregants. Forty-seven of the 48 transformants tested yielded no FOA-resistant segregants, suggesting that *URA3* and *TRP1* are linked in those strains.

Ten transformants in which *TRP1* and *URA3* appeared to be linked were then examined by pulsed-field gel electrophoresis (14, 15) and Southern blot hybridization (16) to determine whether they contained a YAC of the expected size carrying adenoviral DNA sequences. Seven of the transformants produced a single band close to the size of the predicted adenovirus YAC (53 kb) that hybridized to labeled Ad2 virion DNA, two produced the 53-kb band and additional bands of

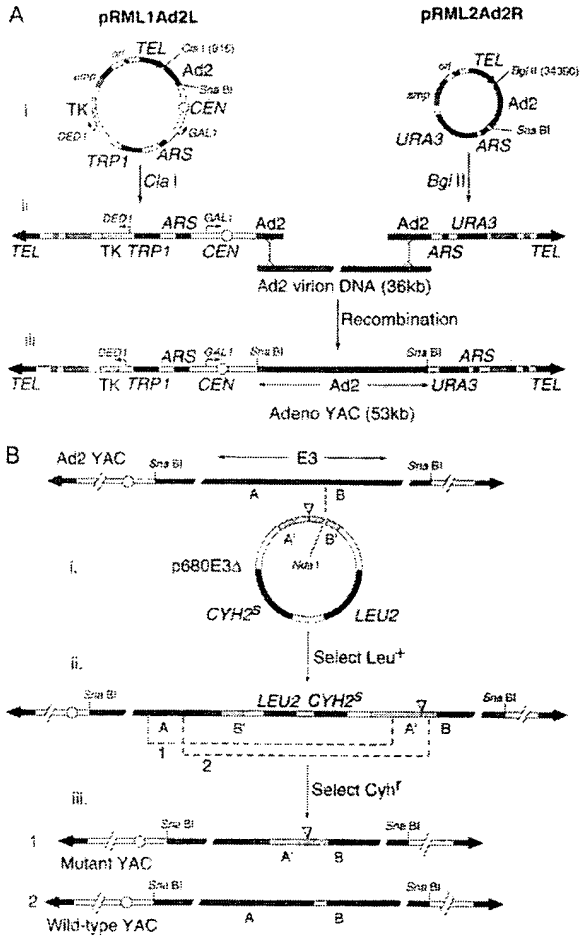

FIG. 1. (*A*) Construction of an adenovirus YAC by recombinational cloning. (*i*) Maps of the plasmids pRML1Ad2L and pRML2Ad2R. (*ii*) Recombination events (}{) required to assemble an adenovirus YAC from Ad2 virion DNA and linear pRML1Ad2L and pRML2Ad2R DNAs. (*iii*) Map of the expected adenovirus YAC. *CEN*, centromere; *TEL*, telomere; *ARS*, yeast autonomously replicating sequence; *amp* and *ori*, ampicillin-resistance gene and plasmid origin of replication; TK, herpes simplex virus thymidine kinase gene; *DED1* and *GAL1*, yeast *DED1* and *GAL1* promoters; *Cla* I, *Bgl* II, and *Sna*BI, restriction enzyme cleavage sites. The positions of the internal restriction sites used to clone the adenovirus segments are indicated. (*B*) Construction of an adenovirus E3 deletion mutant by two-step gene replacement. YAC-containing yeast strains are transformed with p680E3Δ linearized at an *Nde* I site within the Ad2 sequences. Recombination between the YAC and the plasmid at the site of linearization (dashed line) (*i*) integrates the plasmid into the YAC, producing a YAC with two copies of E3 bracketing the remaining plasmid sequences (*ii*). Homologous recombination between the duplicated portions of E3 (dashed lines) can excise the plasmid leaving a single copy of E3 in the YAC. Depending upon where the recombination event occurs, either a mutant or a wild-type copy remains (*iii*). Excision also removes the plasmid *LEU2* and *CYH2r* genes. A, A′ and B, B′ are homologous segments flanking the region of E3 deleted in p680E3Δ. ▽, Deletion mutation. The origin (plasmid or YAC) of the E3 sequences is indicated by shading.

90–150 kb, and the final strain produced a faint band of ≈150 kb (not shown). DNA prepared (6) from the seven strains containing only a 53-kb band produced the bands expected from an adenovirus YAC of the proposed structure after digestion with *Afl* II and *Sna*BI or with *Afl* II alone and analysis by Southern blot hybridization (not shown). These data are consistent with the interpretation that nearly all of the original *TRP1 URA3* transformants contain an adenovirus YAC with the structure diagramed in Fig. 1*A*, created by homologous recombination between viral sequences carried on the linearized YAC vector plasmids and Ad2 virion DNA.

Adenovirus YAC DNA Is Infectious. High molecular weight DNA was prepared from three YAC-containing strains after amplification. As judged by comparison of the intensity of the amplified YAC band to that of the smallest yeast chromosome on a pulsed-field electrophoresis gel (Fig. 2), amplification in these strains is consistently ≈10-fold. The DNA extracted from each strain was digested with *Sna*BI to excise the viral genome from the YAC and the digested DNAs were introduced into 293 cells (17), an adenoviral host cell line, by calcium phosphate transfection (18). Adenoviral plaques arose on transfected dishes at a frequency of 2–10 plaques per μg of total yeast DNA. By assuming 10 copies of the adenovirus YAC per cell after amplification, this corresponds to a specific infectivity of 100–500 plaque-forming units/μg of viral DNA, comparable to that obtained for deproteinized Ad2 virion DNA in parallel transfections. The viral DNA produced by cleavage of a YAC with *Sna*BI will contain a 3-base remnant of the *Sna*BI site at each end; apparently, these extra bases do not substantially reduce the infectivity of the excised genome. No plaques have been observed on plates transfected with a total of 20 μg of undigested DNA. Thus, *Sna*BI digestion increases the infectivity of adenovirus YAC DNA at least 40- to 200-fold.

Construction of an Adenovirus Mutant by Two-Step Gene Replacement. A variety of techniques have been developed for the genetic manipulation of YACs and natural chromosomes in yeast cells. One of these, two-step gene replacement, exploits targeted homologous recombination in transformed cells to replace a selected segment of yeast DNA with a mutant or exogenous sequence (19). To confirm the suitability of the adenovirus YAC for manipulation by such techniques, two-step gene replacement was used to introduce an early region 3 (E3) deletion mutation into the viral DNA. An Ad2 DNA segment encompassing E3 (Ad2 nt 27,410–31,825, with sequences from nt 28,404 to 30,801 replaced by a polylinker) was constructed in p680, a plasmid specifically designed for use in two-step gene replacements in YACs in conjunction with the *leu2* and *cyh2ʳ* markers present in YPH857. The resulting plasmid, p680E3Δ (Fig. 1*Bi*) was linearized by cleavage at a unique *Nde* I site in E3 (Ad2 residue 31,076) and was introduced into a YAC-containing strain. *LEU2* transformants were selected. Linearization of p680E3Δ within the Ad2 sequence targets recombination to that site, and transformants should contain YACs with a complete copy of p680E3Δ integrated at the Ad2 E3 *Nde* I site. These YACs will carry two copies of E3, one wild-type and one mutant, separated by an integrated copy of the p680 plasmid (Fig. 1*Bii*).

YPH857 (*cyh2ʳ*) is resistant to cycloheximide. However, p680 includes the dominant sensitivity allele (*CYH2ˢ*) of *CYH2* (9) and strains carrying a YAC with an integrated copy of p680E3Δ are cycloheximide-sensitive. During growth of such strains, spontaneous homologous recombination events between the tandemly duplicated E3 segments can excise the p680 sequences and one copy of E3 from the YAC. Those events restore cycloheximide resistance and, depending upon the site at which recombination occurs, generate a YAC bearing either wild-type E3 or a deletion mutant derivative (Fig. 1*Biii*). Thus, E3 mutant YACs should be found among cycloheximide-resistant segregants of strains transformed by p680E3Δ. Several *LEU2* transformants were grown overnight in YPD broth to permit excision of the plasmid by recombination, and 100-μl portions of the cultures were plated on medium that selects for *URA3*, *TRP1*, and *cyh2ʳ*. Sixteen segregants derived from three transformants were examined by Southern blot hybridization (Fig. 3). Ten contained a wild-type YAC and 4 carried E3 deletion mutant

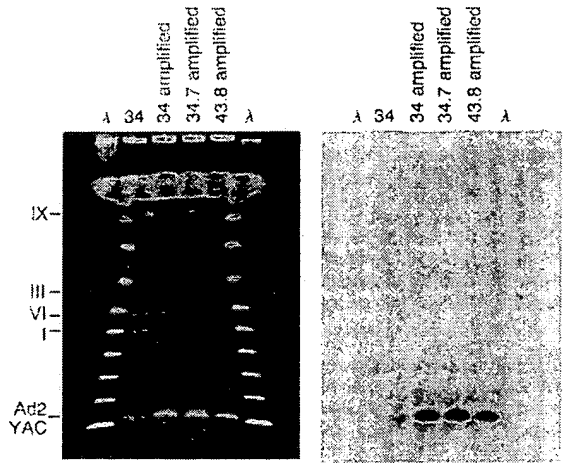

FIG. 2. Pulsed-field gel analysis of amplified YAC DNA. DNAs prepared from YAC strain 34, which contains a wild-type YAC, from strain 34 after amplification of the YAC, and from two strains (34.7 and 34.8) carrying E3 deletion mutant YACs after amplification, were fractionated by pulsed-field gel electrophoresis (270 V; 10 h; switching time, 15 s). The ethidium bromide-stained gel (*Left*) and an autoradiogram prepared after Southern blot hybridization (*Right*) are shown. The positions of the four smallest yeast chromosomes and of the adenovirus YAC are marked on the left. λ, λ DNA concatemer ladder.

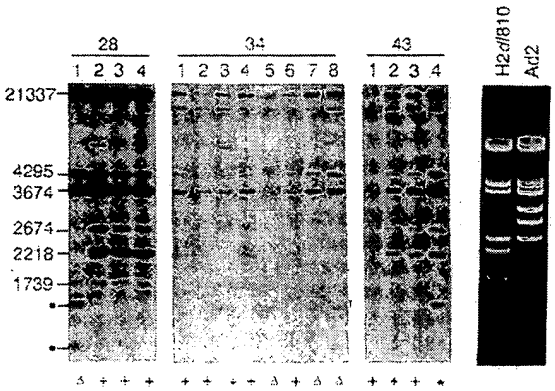

FIG. 3. (*Left*) Identification of E3 deletion mutant YACs after two-step gene replacement. DNAs prepared from 16 strains generated by the two-step gene replacement protocol from p680ΔE3 and YAC strains 28, 34, and 43 were digested with *Eco*RI and examined by Southern blot hybridization. Symbols beneath each lane of the autoradiograms indicate the genotype of the YAC present in the corresponding strain: Δ, E3 deletion mutant; +, wild type; •, YAC retaining the E3 duplication and an integrated copy of p680. The positions and sizes of the *Eco*RI fragments of wild-type Ad2 are indicated on the left; the bullets indicate the two new fragments produced by *Eco*RI digestion of E3 deletion mutant DNA. (*Right*) Ethidium bromide-stained agarose gel displaying *Eco*RI restriction fragments of H2*dl*810, an E3 mutant virus derived from YAC strain 34.7, and wild-type Ad2.

YACs. Two strains carried YACs that retained the integrated p680E3Δ sequences and may have become cycloheximide-resistant by mutation or conversion of *CYH2*^{r}.

Viruses were recovered from two strains carrying deletion mutant YACs by transfection of 293 cells with amplified *Sna*BI-digested DNA. All DNAs prepared from 21 viral isolates examined by restriction enzyme digestion and gel electrophoresis showed the restriction pattern expected for an E3 mutant (data not shown). These experiments confirm that the adenovirus YAC can be manipulated by conventional yeast genetic techniques and that viral mutants can be readily recovered from a modified YAC.

Construction of an Adenovirus Expression Vector. Adenoviruses have been used as vectors for the expression of foreign DNAs in cells and whole animals (4, 5). To demonstrate the feasibility of producing vectors by manipulation of the adenovirus YAC, an adenovirus derivative that expresses an exogenous gene was constructed. The p680E3Δ polylinker replaces the majority of E3 coding sequences (ref. 20; Fig. 4*A*), and adenovirus mutants containing insertions at that site should express the inserted DNA under the control of E3 regulatory sequences. An epitope-tagged version of the human homolog of the yeast *CDC27* gene (CDC27Hs; ref. 10) was inserted in the p680E3Δ polylinker, the resulting E3::CDC27Hs substitution mutation was introduced into the adenovirus YAC by two-step gene replacement, and a virus containing the substitution (H2*sub*CDC27Hs) was recovered. Extracts prepared 22 h after infection of HeLa cells by H2*sub*CDC27Hs were examined by SDS/PAGE and immunoblot analysis using polyclonal antisera directed against either the epitope tag or bacterially expressed CDC27Hs. Both antisera detected a protein at the expected position of epitope-tagged CDC27Hs in cells infected with H2*sub*-CDC27Hs but not in cells infected with wild-type adenovirus

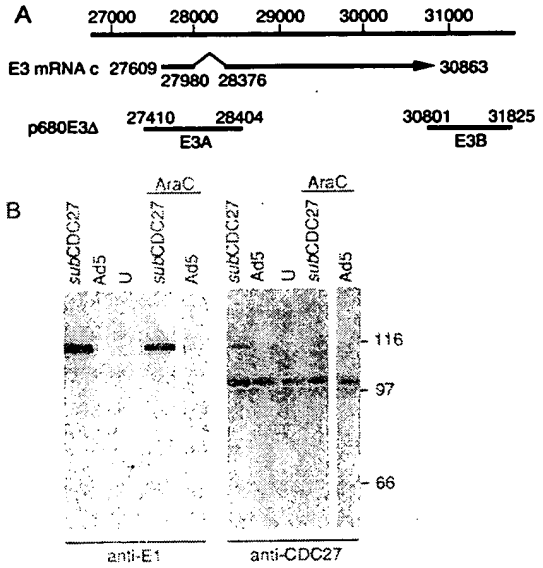

FIG. 4. Expression of the human *CDC27* gene by H2*sub*-CDC27Hs. (*A*) The positions of the E3 cap and polyadenylylation sites and the common E3 splice donor and acceptor sites are indicated on a diagram of E3 mRNA c (20). The adenoviral segments present in p680E3Δ (E3A and E3B) are shown below. Numbers refer to nucleotide position on the Ad2 genome. (*B*) Extracts from HeLa cells infected with H2*sub*CDC27Hs or with adenovirus type 5 (Ad5) and extracts from uninfected cells were fractionated on an SDS/10% polyacrylamide gel, transferred to a nylon membrane filter, and probed with antisera directed against the epitope tag E1 or the intact protein CDC27Hs.

or in uninfected cells (Fig. 4*B*). Material reactive with both antisera was also found in cells infected with H2*sub*-CDC27Hs and incubated in the presence of cytosine arabinoside, which blocks the replication of adenoviral DNA and restricts expression of the viral genome to early regions (21). The antiserum against the intact protein reacted with the tagged CDC27Hs in H2*sub*CDC27Hs-infected cells and with endogenous CDC27 present all extracts. These data demonstrate that H2*sub*CDC27Hs expresses the CDC27Hs gene and suggest that the expression of CDC27Hs is driven at least in part by E3 regulatory signals.

Recombinationally Targeted Cloning. The recombinational cloning method used to construct the adenovirus YAC could in principle be applied to the cloning of any DNA segment as a YAC if DNA fragments that bracket the target segment can be obtained. Further, the extremely efficient production of adenovirus YACs in our original experiments suggested to us that it might be possible to use recombination *in vivo* to clone specific segments of DNA from sources such as the genomic DNA of higher organisms, in which the target is a minor component. To determine the efficiency of YAC formation from a rare target segment, a series of experiments was performed in which decreasing amounts of Ad2 DNA mixed with mouse DNA carrier were introduced, along with linearized pRML1Ad2L and pRML2Ad2R DNAs, into YPH857 spheroplasts. The total amount of the DNA mixture (virus plus mouse) was kept constant, while the proportion of viral DNA in the mixture was varied from exclusively Ad2 to $1/10^5$ by mass. The latter is approximately equivalent to one copy of the viral genome (3.6×10^4 bp) per mouse haploid genome equivalent (3×10^9 bp). *TRP1 URA3* transformants arising from each transformation were tested for stability of *URA3* under selection for *TRP1*, for the presence of Ad2 DNA sequences by colony hybridization to an internal Ad2 probe (22), and for the presence of an adenovirus YAC by pulsed-field electrophoresis and conventional Southern blot hybridization (Table 1).

The frequency of transformation to *TRP1 URA3* was independent of the amount of adenovirus DNA present in the transformation. The number of transformants in which *URA3* was stable under selection for *TRP1* decreased with decreasing Ad2 DNA to a minimum of ≈25% of transformants at a

Table 1. Efficiency of recombinationally targeted cloning

Ad2 DNA	*URA3* stable	Fraction: Hybridization to Ad2 probe	Fraction: Authentic Ad2 YACs	Equivalent genome
1	0.96			
10^{-1}	0.75	0.98 (52)		
10^{-2}	0.56			*E. coli*
10^{-3}	0.24	0.07 (156)		*C. albicans*
10^{-4}	0.31	0.007 (444)	3/3	*Drosophila*, *C. elegans*
10^{-5}	0.25	None (384)		Mammals
None	0.23			

YPH857 spheroplasts were transformed with linearized pRML-1Ad2L (2.8 μg) and pRML2Ad2R (1.2 μg) DNAs and with a total of 6.7 μg of a mixture of Ad2 and mouse DNAs. In different transformations, the fractions of viral DNA in this mixture varied from 1 to 10^{-5} (as indicated). The fraction of transformants in which *URA3* was mitotically stable under selection for *TRP1*, the fraction that hybridized to an internal Ad2 DNA fragment, and the proportion of hybridization-positive strains that harbored an authentic adenovirus YAC (judged by size or structure) were determined. For the organisms listed in the final column, a single-copy 36-kb DNA segment constitutes a fraction of the haploid genome equal to or larger than the fraction of the input DNA made up of Ad2 in the corresponding transformation. Number of transformants screened is in parentheses. *C. albicans*, *Candida albicans*; *C. elegans*, *Caenorhabditis elegans*.

level of viral DNA corresponding to ≈100 viral DNA molecules per mouse genome equivalent (Table 1, line 4) and remained constant at lower levels and in the absence of Ad2 DNA. The fraction of transformants that hybridized to the Ad2 probe also decreased with decreasing input Ad2 DNA to a frequency of 0.7% at 10 viral DNA molecules per mouse genome equivalent (line 5). No hybridizing transformants were detected among 384 colonies screened in the transformation performed at 1 viral DNA molecule per mouse genome equivalent (line 6). Of the three strains from the 10-copy transformation identified by colony hybridization as containing viral DNA, all contained a YAC of the expected size and/or with the expected structure as assessed by Southern blot hybridization (data not shown). It is therefore possible with modest effort to recover segments of DNA by recombinational YAC cloning at levels of representation as low as 10 copies per mammalian genome equivalent.

DISCUSSION

Recombination-based methods for the manipulation of the adenoviral genome in yeast have several advantages over conventional methods for the construction of mutants: they are applicable to any region of the viral genome, they are independent of the placement of restriction sites in the viral DNA and, since microbiological cloning is accomplished in the yeast host, they eliminate the need for time-consuming plaque purification of mutant virus. Because constructions involving several successive modifications of the viral genome can be carried out without the isolation of viral intermediates, such methods will be particularly useful in the preparation of multiple mutants and complex viral vectors for gene delivery. It should be noted that mutant virus containing internal *Sna*BI sites cannot easily be produced from the existing adenovirus YAC.

The recombinational strategies used to make and manipulate the adenovirus YAC have obvious application to other viral genomes. For example, we have used recombinational cloning to construct YAC clones of simian immunodeficiency virus (SIV) and human immunodeficiency virus and have produced a SIV mutant by two-step gene replacement (D. Hauer, G.K., and J. E. Clements, unpublished data). YACs containing cDNA copies of conventional RNA viruses could also be constructed. For each viral YAC, a method for producing infectious nucleic acid is required. For the adenovirus YAC, infectious DNA can be excised from the YAC by restriction enzyme digestion, and the SIV YAC is itself infectious. For large viral DNAs, it may be possible to use an excisional strategy and a nuclease with a long recognition sequence. For positive-strand viruses whose genomic RNA is infectious, transcription of the cDNA from an appropriately positioned yeast promoter might produce infectious viral RNA in the yeast cell. It may even be possible to extend the technique to negative-strand RNA viruses by coproducing the viral replicase and genome-like negative-strand RNA in yeast and by transferring both components to animal cells by spheroplast fusion.

The high efficiency of formation of adenovirus YACs in the original transformations suggested that the targeted production of YAC clones of specific segments from complex DNAs could be accomplished by recombinational cloning. In reconstruction experiments that used small amounts of adenoviral DNA in large excesses of mammalian DNA to simulate rare DNA segments, it proved possible to recover adenovirus YACs from mixtures that contained as few as 10 copies of adenoviral DNA per haploid mammalian genome equivalent. Because the efficiency of recombinational cloning may be affected by factors such as the size of the target and position of the physical ends of the target segment, the data obtained in the reconstruction may only approximate the results that can be expected when cloning a segment from genomic DNA. Nevertheless, the recovery of adenovirus YACs from DNA mixtures containing very small proportions of Ad2 DNA strongly indicates that for genomes of lower complexity than that of the mouse (such as *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Candida albicans*), single-copy genes can be obtained directly by recombinationally targeted YAC cloning. In the reconstruction experiment, YAC recovery declined in a roughly linear fashion with decreasing Ad2 DNA content, suggesting that single-copy segments in mammalian DNA may be recoverable simply by increasing the screening effort (the expected yield is ≈1 YAC per 1500 transformants). Alternatively, enrichment of target sequences (for example, by pulsed-field gel electrophoresis) or refinement of the technique (for example, optimization of the ratios of YAC vector plasmids to target DNA) may permit more efficient recovery of YACs from mammalian DNA. The ability to rapidly produce YAC clones of specific segments of a mammalian genome could be of value for several applications including the isolation of genomic clones of multiple allelic variants of a cloned gene, for obtaining cognates of cloned genomic regions from related species, or for obtaining clones covering gaps in existing libraries.

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